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TITLE OF INVENTION

HCV/BVDV Chimeric Genomes and Uses Thereof

FIELD OF INVENTION

The present invention relates to molecular approaches to the production of nucleic acid sequences which comprise the genomes of chimeric hepatitis C virus-bovine viral diarrhea viruses (HCV-BVDV). The invention also relates to the use of these chimeric nucleic acid sequences to produce chimeric virions in cells and the use of these chimeric virions in HCV antibody neutralization assays, and for the development of vaccines and therapeutics for HCV.

Background Of Invention

Hepatitis C virus (HCV) has a positive-sense single-strand RNA genome and is a member of the genus Hepacivirus within the Flaviviridae family of viruses (Rice, 1996). As for all positive-stranded RNA viruses, the genome of HCV functions as mRNA from which all viral proteins necessary for propagation are translated.

The viral genome of HCV is approximately 9600 nucleotides (nts) in length and consists of a highly conserved 5' untranslated region (UTR), a single long open reading frame (ORF) of approximately 9,000 nts and a complex 3' UTR. The 5' UTR contains an internal ribosomal entry site (Tsukiyama-Kohara et al., 1992; Honda et al., 1996). The 3' UTR consists of a short variable region, a polypyrimidine tract of variable length and, at the 3' end, a highly conserved region of approximately 100 nucleotides (Kolykhalov et al., 1996;

Tanaka et al., 1995; Tanaka et al., 1996; Yamada et al., 1996). The last 46 nucleotides of this conserved region were predicted to form a stable stem-loop structure thought to be critical for viral replication (Blight and Rice, 1997; Ito and Lai, 1997; Tsuchihara et al., 1997). The ORF encodes a large polypeptide precursor that is cleaved into at least 10 proteins by host and viral proteinases (Rice, 1996). The predicted envelope proteins contain several conserved N-linked glycosylation sites and cysteine residues (Okamoto et al., 1992a). The NS3 gene encodes a serine protease and an RNA helicase and the NS5B gene encodes an RNA-dependent RNA polymerase.

A remarkable characteristic of HCV is its genetic heterogeneity, which is manifested throughout the genome (Bukh *et al.*, 1995). The most heterogeneous regions of the genome are found in the envelope genes, in particular the hypervariable region 1 (HVR1) at the N-terminus of E2 (Hijikata *et al.*, 1991; Weiner *et al.*, 1991). HCV circulates as a quasispecies of closely related genomes in an infected individual. Globally, six major HCV genotypes (genotypes 1-6) and multiple subtypes (a, b, c, etc.) have been identified (Bukh et al., 1993; Simmonds et al., 1993).

The nucleotide and deduced amino acid sequences among isolates within a quasispecies generally differ by < 2%, whereas those between isolates of different genotypes vary by as much as 35%. Genotypes 1, 2 and 3 are found worldwide and constitute more than 90% of the HCV infections in North and South America, Europe, Russia, China, Japan and Australia (Forns and Bukh, 1998). Throughout these regions genotype 1

accounts for the majority of HCV infections but genotypes 2 and 3 each account for 5-15%.

At present, more than 80% of individuals infected with HCV become chronically infected and these chronically infected individuals have a relatively high risk of developing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997). The only effective therapy for chronic hepatitis C, interferon (IFN), alone or in combination with ribavirin, induces a sustained response in less than 50% of treated patients (Davis et al., 1998; McHutchinson et al., 1998). Consequently, HCV is currently the most common cause of end stage liver failure and the reason for about 30% of liver transplants performed in the U.S. (Hoofnagle, 1997). In addition, a number of recent studies suggested that the severity of liver disease and the outcome of therapy may be genotype-dependent (reviewed in Bukh et al., 1997). In particular, these studies suggested that infection with HCV genotype 1b was associated with more severe liver disease (Brechot, 1997) and a poorer response to IFN therapy (Fried and Hoofnagle, 1995). As a result of the inability to develop a universally effective therapy against HCV infection, it is estimated that there are still more than 25,000 new infections yearly in the U.S. (Alter 1997) Moreover, since there is no vaccine for HCV, HCV remains a serious public health problem.

Despite the intense interest in the development of vaccines and therapies for HCV, progress has been hindered by the absence of a useful cell culture system for laboratory study (2-7). For example, although the virus has been grown in some cell lines,

the level of replication is so low that RT-PCR assays are required for virus detection; these RT-PCR assays, especially those for negative strand RNA, are tedious and prone to artifacts, and the results have been difficult to reproduce.

Summary Of The Invention

The present invention relates to chimeric nucleic acid sequences which comprise the genomes of chimeric hepatitis C virus-bovine viral diarrhea viruses (HCV-BVDV). More specifically, the chimeric viruses are produced by replacing the structural region or a structural gene of a bovine viral diarrhea virus (BVDV) with the corresponding region or gene of an infectious hepatitis C virus (HCV).

The present invention also relates to the in vitro and in vivo production of chimeric HCV/BVDV viruses from the chimeric nucleic acid sequences of the invention.

The present invention also relates to the use of the chimeric viruses of the invention to identify cell lines capable of supporting the replication of the chimeric viruses.

The invention further relates to the use of the chimeric viruses of the invention to screen for neutralizing antibodies to HCV of different genotypes.

The invention also relates to the use of the chimeric nucleic acid sequences of the invention in the production of HCV-BVDV virions, and the use of these HCV-BVDV virions for the development of inactivated or attenuated vaccines to prevent HCV-BVDV in a mammal.

The invention also relates to the use of the

chimeric nucleic acid sequences to study the molecular properties of HCV indirectly in vitro.

The present invention also relates to the polypeptides encoded by the chimeric nucleic acid sequences of the invention or fragments thereof.

The invention also provides that the chimeric nucleic acid sequences and the chimeric viruses of the invention be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

DESCRIPTION OF FIGURES

Fig. 1. Genomic organization of BVDV, HCV and HCV/BVDV chimera. The BVDV and HCV are NADL (14, 21) and H77 strains (12), respectively. The complete BVDV-NADL genome consists of, in 5' to 3' order, 5'NCR (nucleotides 1-385), N^{pro} (nucleotides 386-889), Core (nucleotides 890-1195), E^{rns} (nucleotides 1196-1876), E1 (nucleotides 1877-2461), E2 (nucleotides 2462-3583), P7 and nonstructural genes (nucleotides 3584-12349) and 3'NCR (nucleotides 12352-12578).

Fig. 2. Strategy for the construction of chimeric cDNA, pHCV/BVDV-3, which has core, E1 and E2 of HCV in the backbone of BVDV. The fusion PCR products were cloned into pBV18-F2 after digestion with *SnaB* I and *Bsm* I. The fragments containing fusion PCR products were cloned into pSDMlu-3' after digestion with *Cla* I and *Dra* III.

Figures 3A-3H show the nucleotide and deduced amino acid sequences of the infectious HCV clone of genotype 1a.

Figures 4A-4H show the nucleotide and deduced amino acid sequences of the infectious clone of genotype

1b.

Figure 5 shows a Western blot of lysate and supernatant from EBTr(A) cells infected with chimeric HCV-BVDV clone pHCV-BVDV-3 using antibody to HCV E1, E2 or core proteins.

DESCRIPTION OF THE INVENTION

The present invention relates to nucleic acid sequences which comprise the genomes of chimeric HCV-BVDV. The chimeric viruses are produced by replacing the structural region or a structural gene (or fragment thereof) of a bovine viral diarrhea virus (BVDV) with the corresponding region or gene (or fragment thereof) of an hepatitis C virus (HCV). The gene borders of the HCV genome, including nucleotide and amino acid locations, have been determined, for example, as depicted in Houghton, M. (1996), and the putative gene borders of the BVDV genome are shown in Figure 1.

In one embodiment, the chimeric nucleic acid sequence comprises the structural genes from an infectious HCV clone and the nonstructural genes and untranslated regions from an BVDV clone.

In another embodiment, additional HCV/BVDV chimeras can be constructed to study HCV infection of cell lines. For example, additional HCV/BVDV chimeras may be made in which only E1 and E2 genes of the BVDV infectious clone are replaced with the corresponding genes from an HCV clone. Such chimeras can be used to determine whether the core protein of BVDV is critical for encapsidation of the viral RNA. Alternatively, HCV/BVDV chimeras in which either the E1 or E2 gene of BVDV is replaced by the corresponding gene of HCV may be

constructed. Such chimeras can be used to determine the relative importance of E1 or E2 for infection of cell lines. In another embodiment, HCV/BVDV chimeras in which one of the nonstructural genes of BVDV, such as NS3 RNA helicase, NS3 protease, or the NS5B RNA-dependent RNA polymerase are replaced by the corresponding non-structural genes of HCV may be constructed. Such chimeras would, for example, be useful in identifying inhibitors of viral enzyme activity which would be useful as antiviral agents.

In yet another embodiment, hypervariable region 1 (HVR1) from multiple HCV genotypes may be combined into one HCV/BVDV chimera. The only limit for constructing this type of chimera is that the viral genome must be able to be packaged. Alternatively, a chimera can be constructed which contain an HVR1 sequence from one HCV genotype. Such chimeras can be used as an inactivated multivalent vaccine or to screen for neutralizing antibodies to multiple HCV genotypes.

The HCV/BVDV chimeras of the invention may be constructed using any HCV and BVDV clones. However, in a preferred embodiment, the HCV clones are infectious HCV clones of genotype 1a (ATCC accession number PTA-157; Figures 3A-3F), 1b (ATCC accession number 209596; Figures 4A-4F) or 2a (ATCC accession number PTA-153; SEQ ID NOS:3-4) and the infectious BVDV clone pVVNADL are used.

In constructing the chimeric nucleic acid sequences of the invention, it is to be understood that the retention of the E^{rns} gene of BVDV in any chimeric is entirely optional. Thus, when it is stated that the HCV/BVDV chimeras could be constructed in which, for

example, the E1 or E2 gene of BVDV is replaced by the corresponding E1 or E2 gene of HCV, it is to be understood that the resultant chimeras may or may not retain the BVDV E^{rns} gene.

The present invention further relates to the production of chimeric HCV/BVDV viruses from the HCV/BVDV chimeras of the invention.

In one embodiment, the chimeric sequences of the invention can be inserted into an expression vector that functions in eukaryotic cells. Such eukaryotic expression vectors are well known to those of ordinary skill in the art and include, but are not limited to, plasmids, vaccinia viruses, retroviruses, adenoviruses and adeno-associated viruses.

The sequences contained in the recombinant expression vector can then be transcribed in vitro by methods known to those of ordinary skill in the art in order to produce RNA transcripts which encode the chimeric viruses of the invention. The chimeric viruses of the invention may then be produced by transfecting cells by methods known to those of ordinary skill in the art with either the in vitro transcription mixture containing the RNA transcripts or with the recombinant expression vectors containing the nucleic acid sequences described herein.

Where transfection of cells with recombinant expression vectors containing the nucleic acid sequences of the invention is used, transfection may be done by methods known in the art such as electroporation, precipitation with DEAE-Dextran or calcium phosphate, or incorporation into liposomes.

In one such embodiment, the method comprises

the growing of animal cells in vitro and transfecting the cells with the chimeric nucleic acid of the invention, then determining if the cells show indicia of HCV infection. Such indicia include the detection of viral antigens in the cell, for example, by immunofluorescence procedures well known in the art; the detection of viral polypeptides by Western blotting using antibodies specific therefor; and the detection of newly transcribed viral RNA within the cells via methods such as RT-PCR. The presence of live, infectious virus particles following such tests may also be shown by injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the signs and symptoms of HCV infection. Alternatively, the presence of live, infectious virus particles following such tests may also be shown by serial passaging the chimeric virus in cells.

Suitable cells or cell lines for culturing the chimeric viruses of the invention include, but are not limited to, EBTr(A) and Huh7.

Preferably, transfection of cells with the chimeric sequences is carried out in the presence of helper BVDV which is preferably of a noncytopathogenic strain. In one embodiment, the cell lines to be infected may already contain a helper BVDV. Such cells include, but are not limited to, EBTr(A). Alternatively, the cell lines to be transfected may be infected with a helper BVDV prior to, or concurrent with, transfection with the chimeric sequences of the invention.

The present invention also relates to polypeptides encoded by the chimeric nucleic acid

sequences of the invention or fragments thereof. In one embodiment, said polypeptide or polypeptides may be fully or partially purified from viruses produced by cells transfected with the chimeric nucleic acid sequences of the invention. In another embodiment, the polypeptide or polypeptides may be produced recombinantly from a fragment of the chimeric nucleic acid sequences of the invention. In yet another embodiment, the polypeptides may be chemically synthesized.

The present invention also relates to the use of the chimeric sequences of the invention to identify cell lines capable of supporting the replication of the chimeric viruses of the invention.

In another embodiment, the invention relates to the use of HCV/BVDV chimeras to screen for neutralizing antibodies to HCV of different genotypes. For example, chimeric viruses produced in cell lines infected with the chimeric clones of the invention can be used in neutralization assays to test the neutralizing ability of anti-HCV antibodies.

In yet another embodiment, the invention relates to the use of the infectious chimeric clones of the invention to develop inactivated or attenuated vaccines to prevent Hepatitis C in a mammal. For example, chimeric virions from cell lines infected with a chimeric virus of the invention, or transfected with a chimeric sequence of the invention, can be purified from the cells and inactivated by methods known to those of ordinary skill in the art. The inactivated HCV-BVDV virions can be used to immunize mice, and if neutralizing antibody to HCV is produced, the virions

can then be used to immunize chimpanzees to determine whether the antibodies are protective. Alternatively, cells infected with the chimeric viruses of the invention may be passaged in cell culture to produce attenuated viruses which can be tested as candidate live vaccines. In assaying the ability of the chimeric viruses of the invention to infect mammals one can assay sera or liver of the infected mammal by RT-PCR to determine viral titer. In addition, the virulence phenotype of the virus produced by transfection of mammals with the sequences of the invention can be monitored by methods known in the art such as measurement of liver enzyme levels (alanine aminotransferase (ALT) or isocitrate dehydrogenase (ICD)) or by histopathology of liver biopsies.

Alternatively, mutations may be introduced into the HCV portion of the HCV/BVDV chimeras of the invention in order to enable the production of virions in cell cultures which could then be tested in vivo for improved vaccine properties.

In another embodiment, multiple chimeras containing HCV structural genes (or fragments thereof, such as the HVR1) from multiple genotypes can be administered to generate multivalent vaccines.

When used as a vaccine, the chimeric virions can be administered alone or in a suitable diluent, including, but not limited to, water, saline, or some type of buffered medium. The vaccine according to the present invention may be administered to an animal, especially a mammal, and most especially a human, by a variety of routes, including, but not limited to, intradermally, intramuscularly, subcutaneously, or in

any combination thereof. Of course, it is understood that formulations or compositions comprising the chimeric virions of the invention may be used either therapeutically or prophylactically to treat or prevent the signs and symptoms of HCV.

The present invention therefore also relates to antibodies reactive with the HCV structural polypeptide(s) contained in the HCV-BVDV virions of the invention where such antibodies are produced following immunization with the HCV-BVDV virions.

The antibody molecules of the present invention may be polyclonal or monoclonal and may be useful in the prevention or treatment of diseases caused by HCV in mammals.

The invention also provides that the chimeric nucleic acid sequences and the chimeric viruses of the invention be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

All scientific publications and/or patents cited herein are specifically incorporated by reference. The following examples illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

EXAMPLES

Materials and Methods

Cell lines

The different cell lines used in the present study are listed in Table 1. Most cells were grown in Dulbecco's Minimum Essential Medium (DMEM) or DMEM-F12 supplemented with horse serum or with irradiated fetal calf serum. In some cases, Boyt serum, a fetal calf

serum free of BVDV and antibodies to BVDV (Boyt Veternary, Neoshoe, MO) was used. All cells were incubated at 37°C in 5% CO_2.

Table 1

List of Cell Lines

Cell	Origin	Medium
EBTr(A)	Embryonic bovine trachea	10% FBS/MEM
BT	Bovine turbinate	10% horse serum/MEM
MDBK	Bovine kidney	10% horse serum/MEM
EBTr (B)	Embryonic bovine trachea	10% FBS/MEM
Huh 7	human hepatoma	10% FBS/DMEM F12

Antibodies

H79: plasma from patient H obtained in the chronic phase two years after the onset of HCV infection (11); CH1530: serum pool from chimpanzee 1530, obtained in the chronic phase one to two years after the onset of HCV infection. Chimpanzee 1530 became infected with HCV following intrahepatic transfection with pCV-H77C (Yanagi 1997); LMF86 and LMF87: anti-HVR1 (Farci 1996), rabbit anti-peptide sera; Mab NS: anti-BVDV NS3 murine monoclonal antibody kindly provided by Dr. E. Dubovi (Cornell University, Ithaca, NY).

Construction of HCV/BVDV chimeric clone

The C, E1 and E2 genes originating from an infectious clone of the H77 strain of HCV (pCV-H77C, ref. Yanagi 1997), and the backbone originating from two subgenomic plasmids (pBV18-F2 and pSDMlu-3'), used by Vassilev et al. (Vassilev 1997) to generate the infectious clone of the NADL strain of BVDV (pVVNADL), were used to construct the chimeric cDNA clone pHCV-BVDV-3 (ATCC deposit Number PTA-158). The chimeric clone includes sequences corresponding to nucleotides

345-2579 (amino acids 2-746) of the pCV-H77C clone of HCV and nucleotides 1-927 (amino acids 1-168) and nucleotides 3622-14578 (amino acids 1067-3988) of the pVVNADL clone of BVDV (Figure 1). To generate the desired junctions between HCV and BVDV, standard PCR and fusion PCR were performed with pfu polymerase (Strategene) and the oligonucleotides listed in Table 2. One PCR fragment was amplified from pCV-H77C with primers Npro-C/H77/S and E2-P7/H77/R, two other fragments were amplified from pBV18-F2 with primers MluI/NADL/S and Npro-C/NADL/R and with primers E2-P7/NADL/S and BsmI/NADL/R, respectively. Following purification with the QIAquick PCR purification kit (Qiagen), the three PCR products were mixed and a fusion PCR was performed with primers MluI/NADL/S and BsmI/NADL/R. After purification, the fusion PCR product was cloned into pBV18-F2 by using SnaBI and BsmI sites (Fig. 2) and multiple clones were screened by sequence analysis. Finally, a clone with the correct sequence was digested with ClaI and DraIII and the insert was cloned into pSDMlu-3' to generate the full-length chimeric clone, pHCV/BVDV-3 (Fig. 2). This clone was transformed into JM109 competent cells (Promega) and selected on LB agar plates containing 100 µg/ml ampicillin (SIGMA). Several colonies were cultured in LB liquid containing ampicillin at 30°C for 18~20 hrs. After small scale preparation (Qiagen Minipreps DNA Purification Systems), a plasmid preparation with the expected digestion pattern was retransformed to select a single clone, and large-scale preparation of plasmid DNA was performed with a QIAGEN plasmid Maxi kit as described previously (Yanagi 1997). The complete

HCV/BVDV sequence of the final preparation was determined using standard procedures and about 90 specific sense and antisense primers. Clone pHCV/BVDV-3 was apparently stable since the digestion pattern was as expected following retransformation. The complete sequence differed slightly from the published BVDV sequence of the NADL strain (21), but encoded an intact polyprotein.

Table 2

Oligonucleotides used for PCR amplification

Name	Sequences (5' - 3')	Underline
N-C/H77/S	CAAGTTGCAGCACGAATCCTAAACCTCAAAGAA	N OF BVDV-NADL
Mlul/NADL/S	CACGCGTATCGATGAATTCG	Mlu i
B2-P7/NADL/S	AGCGGAGGCGATTCAGTATGGATCAGGGGAAGTG	E2 OF HCV
E2-P7/H77/R	ATACTGAATCGCCTCCGCTTGGGATATGAG	P7 OF BVDV-NADL
N-C/NADL/R	AGGATTCGTGCTGCAACTTGTGACCCATAGAGGG CAGTC	Core OF HCV
BanI/NADL/R	TACCAGGCTGAGAATGCACTGTAAC	Bsm I
2937S-HCBV	CCTTGTCCACCGGCCTCATCCACCTCCACC	
1353S-NADL	CAATTCATGGTATGATGGATGC	
1419S-NADL	AGTGGAACAAGCATGGTTGGTG	
2335-NADL	CCACGTGGACGAGGGCATGCC	
3342R-NADL	CCTGAATCGGCCTTTACCACATCCCCAATC	
1623R-NADL	TTCTTTCCTTTCTTGCAACCTGT	
1590R-NADL	GGGCTATCTCTAGCTTGTGTTAC	
389R-NADL	CCATGTGCCATGTACAGCAGAG	

Transfection of cell lines with transcribed RNA

The plasmid pHCV/BVDV-3 was linearized with SacII (NEB) and treated with T4 DNA polymerase (GIBCO/BRL) to remove the resulting 3' overhang. A truncated form of pHCV/BVDV-3, generated by digestion with HindIII, was used as a negative control. Two micrograms of DNA were transcribed at 37°C for 2 hrs in a 100 µl reaction volume containing 50 U of T7 RNA polymerase (Promega), 10 mM DTT (Promega), 120 U of Rnasin (Promega) and 1 mM rNTPs (GIBCO/BRL). Five microliters of the final reaction mixture was analyzed

by agarose gel electrophoresis and ethidium bromide staining. The RNA of each transcription mixture was extracted with the TRIzol system (GIBCO/BRL) and resuspended in 50 µl of DEPC-treated water, and stored at -80°C.

For transfection, 3-5 µg of RNA was added to 1 ml of Optimem with 15 µl of DMRIE-C (GIBCO/BRL) and incubated with cells for 5 hrs. The Optimem was removed and complete medium was added. Cells were cultured in the presence of the appropriate medium (Table 1) and transfected at 80% confluency either in one well of a 12 well plate (Costar) or in a 60 mm dish (Costar). About 24 hrs prior to immunofluorescent staining, transfected cells were split into 4- or 8- well chamber slides (LAB-TEK).

Serial passage from transfected or infected cells

The supernatant was collected and stored at -80°C. Cells were scraped with 1 ml of supernatant medium and centrifuged. The pellet was taken through three freezing and thawing cycles to lyse the cells. For homologous passages, lysed cells or supernatant (100 - 500 µl) were transferred onto new cells of the same type. For heterologous passages, lysed cells or supernatant from EBTr(A) cells were transferred onto different cell lines. Inoculated cells were incubated at 37°C for two hrs followed by the addition of complete medium. Inoculated cells were incubated at 37°C for 4-12 days.

Immunofluorescent staining
of transfected or infected cells

Cells grown on chamber slides were fixed and permeabilized with cold acetone for 5 min and washed

with phosphate buffered saline (PBS) for 10 min. Thereafter, cells were incubated for 20-60 min at 37°C with primary antibodies diluted in 10% bovine serum albumin (BSA) in PBS. As primary antibodies we used an anti-HCV human plasma sample (H79, 1:100 dilution), an anti-HCV chimpanzee serum (CH1530, 1:100 dilution) and an anti-BVDV NS3 monoclonal antibody (Mab-NS, 1:10 dilution). After washing with PBS for 15 min, cells were incubated for 20-40 min at 37°C with secondary antibodies; fluorescein-isothiocyanate (FITC)-conjugated goat anti-human antibody (SIGMA) for H79 and CH1530, and rhodamine-conjugated anti-mouse antibody (PIERCE) for anti-BVDV NS3. For double staining, H79 or CH1530 anti-HCV antibody was mixed with the anti-BVDV NS3 monoclonal antibody and incubated on fixed cells as above, followed by washing and incubation with a mixture of both secondary antibodies. After washing, slides were mounted and examined by fluorescence microscopy (Zeiss).

Determination of sucrose
gradient density of recovered viruses

A T150 flask of EBTr(A) cells was inoculated with virus stock. At days 9 and 13, respectively, supernatant was harvested. A total of 70 ml of supernatant was layered over 20% sucrose in TN buffer [50mM Tris and 100mM NaCl (pH 7.4)] and centrifuged at 28,000 rpm in an SW28 swinging bucket rotor (Beckman) for 19 hrs at 4°C. The pellet was resuspended in 100 µl of TN buffer. For sucrose equilibrium gradient centrifugation, the resuspended pellet was layered onto a 20-60% (wt/wt) sucrose gradient in TN buffer and centrifuged at 36,000 rpm in an SW40 swinging bucket

rotor (Beckman) for 20 hrs at 4°C. Fractions of 500 µl each were collected from the bottom of the tube, and the density was determined by refractometry. Finally, the different fractions obtained by RT-PCR were tested for the presence of chimeric HCV/BVDV genomes.

Western blotting for HCV structural proteins

For immunoblotting analysis, EBTr(A) cells (60 mm dish) infected with the HCV/BVDV chimeric virus were lysed by adding 300 µl of M-PER mammalian protein extraction reagent (PIERCE). The cell lysate was cleared of cell debris by low speed centrifugation at 13000 rpm for 5 min. Also, thirty ml of the supernatant from EBTr(A) cells infected with chimeric virus stock was harvested, ultra centrifuged and tested for HCV proteins by immunoblot. Thirty ml of supernatants was layered over 20% sucrose in TN buffer and centrifuged at 28,000 rpm in a SW28 swinging bucket rotor (Beckman) for 19 hrs at 4°C. The pellet was resuspended with 50 ul of TN buffer and 20 ul was assayed by immunoblot. For separation of proteins, 20 µl of lysate or pellet was electrophoresed through a sodium dodecyl sulfate- 12% polyacrylamide gel (SDS-PAGE) and then proteins were transferred onto a nitrocellulose membrane (NOVEX). The membrane was incubated at 4°C for 16 hrs with CH1530 anti-HCV (1:750 dilution) in a blocking buffer containing 1% BSA in TBST buffer (20mM Tris-HCl, pH 7.5; 150mM NaCl, 0.05% Tween 20). Following washing with TBST buffer, the membrane was incubated at room temperature for 1 hr with a 1:5000 dilution of goat anti-human immunoglobulin conjugated to horseradish peroxidase (PIERCE). After washing, the membrane was

incubated with ECL Western blotting detection reagent (Amersham) and exposed to film.

Detection of chimeric genomic RNA by RT-PCR assays

Total RNA was extracted with the TRIzol reagent from 10 or 100 µl of cell suspension, supernatant or material from the sucrose gradient. The RNA pellet was resuspended in 10 mM dithiothreitol (DTT) containing 5% (vol/vol) of RNAsin (20-40 U/µl) (Promega). The RT was performed with avian myeloblastosis virus reverse transcriptase (Promega) and the external anti-sense primer (see below) and PCR was performed with AmpliTaq Gold DNA polymerase (Perkin Elmer) as described (Bukh 1998a). Specificity was confirmed by sequence analysis of selected DNA products. Each set of experiments included a low titer positive control sample and appropriate negative controls. HCV/BVDV chimeric genomes were detected in one round of PCR with the primers 2937S-HCBV and 3342R-HCBV (Table 2). The structural region of BVDV was detected in an RT-nested PCR with external primers 1353S-NADL and 1623R-NADL and internal primers 1419S-NADL and 1590R-NADL (Table 2). These primers were conserved among all known BVDV strains. Finally, the 5' UTR sequence of BVDV was detected by using universal primers that detect both HCV and BVDV (Bukh 1992, Yanagi 1996), as well as universally conserved BVDV primers (233S-NADL and 389R-NADL). The genome equivalent (GE) titer of HCV, BVDV and HCV/BVDV in positive samples was determined by RT-nested PCR on 10-fold serial dilutions of the extracted RNA (Bukh 1998a). One GE was defined as the number of genomes present in the highest dilution

positive in RT-nested PCR. The sensitivity of the RT-PCR assays for HCV/BVDV was established by comparison with the HCV titer determined by using HCV primers with established optimal sensitivity. The consensus sequence of the chimeric HCV/BVDV genome was determined by direct sequencing of overlapping PCR products obtained by long RT-nested PCR on supernatant from infected EBTr(A) cells.

Chimeric virus stocks

Medium harvested from EBTr(A) cells infected with serially passaged chimeric virus was frozen at -80°C and thawed only once.

Focus assay

EBTr(A) cells were trypsinized, diluted in DMEM containing 10% Boyt fetal calf serum, (10% Boyt DMEM) and plated in 2 well chamber slides at 37° C with 5% CO_2 . When the cells were confluent, the medium was aspirated and 200 µl of chimeric virus diluted in 10% Boyt DMEM was added and the cells were gently rocked for 2 hours at room temperature. Cells were then overlaid with 2 ml/well of 0.5% low melting point agarose in minimal essential medium containing 2% fetal calf serum, 20 mM glutamine and 250 µg of gentamicin sulfate/ml. After the agar solidified, the slides were incubated at 37° C and 5% CO_2. After 4 days, the chambers were removed and the agarose discarded. Slides were rinsed briefly in cold phosphate buffered saline (PBS) and immersed in acetone for fixation. Slides were air dried and stained at room temperature for 30 minutes with 100 µl/well of CH1530 anti-HCV serum in diluent containing 1 part 10% bovine serum albumin to 1 part PBS. Slides were washed 5 minutes in PBS. Color development was by

the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) for peroxidase staining per the manufacturer's directions. The peroxidase substrate kit was Vector VIP (Vector Laboratory). Color development was stopped by washing the slide with water followed by air drying. Foci were counted with the aid of a dissecting microscope.

Focus neutralization assay

The assay was performed exactly as for the focus assay except the 200 µl inoculum consisted of 100 µl of chimeric virus diluted in 10% DMEM, 20 µl undiluted test or control serum, and 80 µl 10% DMEM. Each 200 µl sample was incubated at 4° C in ice overnight prior to inoculation of cells. Sera included fetal calf serum (Boyt) and rabbit pre-immune serum as negative controls, hyperimmune rabbit antisera raised to peptides spanning the HVR1 region of the H27 strain of HCV (Farci, 1996), and goat anti-BVDV (VMRD Pullman, WA) prepared without azide. All sera had been heat-inactivated at 56° C for 30 minutes.

Immunofluoresence neutralization assay in Huh7 cells

Two hundred microliters of chimeric virus was mixed with 20 ul of serum or plasma, incubated on ice overnight and added to one well of a four-well chamber slide. After 2 hours at 30°C, 1 ml of agarose overlay was added as for the focus assay. Four days later, slides were fixed and stained as for immunofluoresence microscopy and stained cells were manually counted by scanning the entire well using a Zeis microscope and the 40X objective.

RESULTS

RNA genomes transcribed from the chimeric virus cDNA pHCV-BVDV-3 were transfected into four bovine cell lines, including two independently derived lines of embryonic bovine trachea cells (EBTr). Cells from all four transfected cell lines produced HCV proteins as evidenced by immunofluorescence microscopy using anti-HCV serum (Table 3). However, upon continued incubation, the number of stained cells remained low (< 1 percent) in all except the EBTr(A) line, in which approximately 10 percent of cells stained by day 10. The medium from the transfected EBTr(A) cell line was serially passaged onto new EBTr(A) cells. By the fifth passage, approximately 70 percent of the cells were stained with anti-HCV serum. At passage 5, the entire open reading frame of the genome was amplified by RT-PCR and sequenced. Only one amino acid change, (lys to arg) in the NS3 gene (nucleotide 5373, A to G), was found. This single amino acid mutation coincided with the increased infectivity and may represent an adaptive mutation. After the 10th serial passage in EBTr(A) cells the medium contained 10^8 to 10^9 GE /ml of chimeric genome.

A Western blot of material pelleted from the medium by ultracentrifugation revealed anti-HCV reactive bands consistent in size with core, E1 and E2 proteins of HCV (Figure 5). The chimeric genomes, concentrated by high-speed centrifugation, banded in a sucrose gradient at a density of 1.119 to 1.128 g/ml, suggesting that they were in enveloped virus particles. The sucrose banding pattern, coupled with the Western blot data, suggest that the chimeric genome was enveloped in

a particle containing significant amounts of HCV proteins.

Although the proportion of cells producing HCV proteins increased in EBTr(A) cells, it remained low in the MDBK, BT, and EBTr(B) cell lines, suggesting that the virus was not spreading in these cells. In order to determine if these cells were making infectious virus, a homologous transmission was attempted by removing supernatant from each transfected culture and adding it to a new culture of the same cell line. The only successful transmission was from the transfected EBTr(A) cells to naive EBTr(A) cells (Table 3). Therefore, although the chimeric virus genome could replicate in all four cell lines and produced HCV proteins, only in the EBTr(A) cells was virion morphogenesis coupled with availability of a receptor conducive to infection.

Table 3

Homologous passage and heterologous passage

	Transfection	Homologous passage	Heterologous passage
EBTr(A)	+	+	
EBTr (B)	+	-	+
BT	+	-	+
MDBK	+	-	+

[1] Supernatants from transfected cells were passed onto new cells of the same type.

[2] Supernatants from transfected EBTr(A) cells were passed to indicated cells.

Two heterologous transmission experiments were performed to determine if the three other cell lines released infectious particles. In the first experiment, supernatant from transfected MDBK cells was inoculated onto the EBTr(A) cells. Immunofluorescence microscopy

showed that the cells did not produce HCV proteins, suggesting that infectious particles had not been produced by the transfected MDBK cells. In the second experiment, heterologous transmission of known infectious chimeric virus was attempted. Medium from transfected EBTr(A) cells was inoculated onto cultures of each of the other three cell lines. All of the cell lines became infected, indicating that each displayed a functional receptor for the chimeric virus. Therefore, the MDBK, BT and EBTr(B) cell lines all were permissive for chimeric virus entry and viral genome replication.

The EBTr(A) cells were obtained from the ATCC (ATCC ascession number CLL44) and were not listed as being contaminated with BVDV (9). However, one of the antibodies used to check replication of the chimera was raised against the NS3 protein of the NADL strain of BVDV. At high concentrations this antibody stained, on average, 30-40 percent of uninfected EBTr(A) cells but did not stain EBTr (B) cells, MDBK or BT cells. Therefore, it appeared that EBTr(A) cells either were persistently infected with a noncytopathogenic strain of BVDV, or were harboring a BVDV replicon, or were producing a protein that cross-reacted with the anti-NS3 antibody. To discriminate among these possibilities, medium from naive EBTr(A) cells was inoculated onto MDBK cells. The cells were incubated at 37° C for 4 days and stained with anti-BVDV serum to NS3. A high proportion of the inoculated cells stained with anti-NS3 whereas parallel uninoculated cultures remained negative for NS3 staining. From this result, it was concluded that the EBTr(A) cell line was contaminated with a transmissible agent, most likely a noncytopathogenic strain of BVDV.

RT-PCR primers designed to amplify known BVDV strains were able to amplify a cDNA fragment from uninoculated EBTr(A) cultures (titer: 10^6 GE/ml). The sequence of the cDNA was determined and found to match that of the CP-7 strain of BVDV (18).

Based on the data that only EBTr cells harboring BVDV were able to produce infectious particles containing the chimeric genome, it was hypothesized that the endogenous virus was serving as a helper virus, possibly by providing BVDV structural proteins. In order to determine if the infectious chimeric particles contained BVDV glycoproteins, a focus assay was developed in which cells expressing the chimeric genome were identified by their reactivity with CH1530 anti-HCV serum. An infectivity titer of 10^5 chimeric viruses/ml was obtained for passage 10 virus, which had an RT-PCR titer of 10^8 to 10^9 GE/ml. Chimeric virus produced in EBTr(A) cells was examined for its susceptibility to neutralization by anti-serum to BVDV as compared to neutralization by anti-sera raised against the hypervariable region 1 (HVR1) of the same HCV strain as was in the chimera. Dilutions of chimeric virus were incubated overnight with anti-BVDV, anti-HCV or control sera and the number of infectious particles remaining was determined by the focus assay (Table 4). The number of foci in the rabbit and bovine serum controls decreased in parallel with the dilution factor, indicating that the assay was linear and reliable. The anti-HCV sera did not neutralize the chimera. In contrast, anti-BVDV eliminated all foci at each dilution, suggesting that each and every infectious particle contained BVDV glycoproteins and that they were

probably serving as the viral receptor for binding to the bovine cells. Therefore, the chimeric virus was actually also a pseudotype since the virion contained glycoproteins contributed by a helper virus.

Table 4
Neutralization Assay (Number of Foci/Well)

Virus dilution[1]	Serum				
	LMF86[2]	LMF87[2]	554[3]	FCS[4]	anti-BVDV[5]
1:1000	154[6]	148[6]	168[6]	148[6]	0
1:3,200	65	72	61	61	0
1:10,000	25	29	20	25	0

1. Chimeric virus harvested from the 10th serial passage in EBTr(A)cells.
2. hyperimmune rabbit sera to HVR1 of the H77 strain of HCV.
3. normal rabbit serum.
4. fetal calf serum from Boyt.
5. goat anti-BVDV serum.
6. the number of foci is approximate due to the difficulty in counting overlapping foci.

Western blots of harvested medium described above (Fig. 3) suggested that the HCV glycoproteins were present on the virions in significant amounts even though the chimera was not neutralized by the anti-HVR1 serum.

There are several possible explanations for the failure of the anti-HVR1 serum to neutralize HCV glycoproteins. First, the neutralizing titer may not have been high enough. An earlier bleeding from rabbit LMF87 was able to neutralize 64 chimpanzee infectious doses of the H77 strain of HCV in both of two attempts when tested in chimpanzees. The anti-HVR1 serum we used was from a later bleeding and had not been similarly tested for neutralizing antibodies. Secondly, the HCV glycoproteins might not bind to bovine cells and entry

into these cells might be totally independent of HCV glycoprotein. Thirdly, the HCV E2 glycoprotein might not have folded properly to function or to be recognized by the antibody. The question of the neutralizing potential of the anti-HVR1 serum cannot be answered at this time. By an immunofluoresence microscopy assay, the anti-HVR1 serum had titers of 1:1600 and 1:3200 for rabbits LMF86 and LMF87 respectively but the antibody detected by this assay is not necessarily neutralizing antibody. The functionality of the HCV glycoproteins would best be proved by infecting cells which are not susceptible to infection by BVDV due to an absence of the BVDV receptor. Huh 7 cells were chosen as an experimental system to test for functional HCV glycoproteins because they are a human cell line which grows well and is of hepatocyte origin. Attempts to infect Huh 7 cells with the endogenous BVDV virus of the EBTr(A) cell line were not successful, suggesting either that the receptor for BVDV was absent or that the BVDV genome was unable to replicate in these cells. Attempts to infect the Huh 7 cells with the chimera were more successful. Four days after incubation with 2 X 10^4 EBTr(A) tissue culture infectious doses (TCID) of the chimera, Huh 7 cells could be stained with antibody to NS3 as well as with antibody to HCV. Quantification of the number of infected cells indicated that the inoculum contained 10^3 TCID /ml for Huh 7 as compared to 10^5/ml for EBTr(A) cells. Although the cells could be infected, the virus did not spread, suggesting that in Huh 7 cells , as in the MDBK and BT cells, virions either were not assembled or were not released from cells. Most likely, the CP-7 virus could not provide the

required helper function because it could not enter the cells. This suggested that at least one of the HCV glycoproteins was functioning as a receptor or receptor-component for entry into Huh 7 cells. However, entry into Huh 7 cells was not blocked by preincubation with rabbit LMF87 anti-HVR1 serum but was totally blocked by anti-BVDV serum (Table 5). Therefore, it was possible that one or both of the HCV glycoproteins was functioning in association with one or both of the BVDV glycoproteins, which would have been incorporated during growth in the persistently infected EBTr(A) cells. The failure to neutralize with the anti-HVR1 serum might be due to low neutralizing titers, to mediation of virus entry into these cells by E1 rather than E2 or to interference with antibody binding to the HVR1 because of the BVDV glycoproteins.

The neutralization assay was repeated on Huh 7 cells using human chronic phase plasma (H79) that had, like LMF87 rabbit serum, been shown to neutralize the H72 strain of HCV in a chimpanzee experiment (Farci et al. Proc. Natl. Acad. Sci.USA 91:7792-7796, 1994) (Table 6). Plasmas from 3 chimpanzees that had either been experimentally infected with a monoclonal virus of the H72 strain of HCV (Ch1530 and 1494) or vaccinated with a DNA vaccine expressing the E2 glycoprotein of HCV were also tested. The results suggested that two of the four samples tested contained neutralizing antibodies to HCV. The sample incubated with the human H79 plasma did not infect any cells indicating complete neutralization while the sample incubated with plasma from the DNA vaccinated chimp infected only 1/3 to 1/4 as many cells as the control suggesting it also had neutralizing

antibody but at a lower titer. Since the DNA vaccine expressed only the E2 glycoprotein, this protein must be involved in binding to Huh 7 cells. The plasma from chimp 1530 contained antibodies to the HCV envelope proteins as measured by ELISA or immunofluoresence microscopy but apparently, these were not neutralizing antibodies. Chimpanzee 1494 did not have demonstrable antiodies against the HCV glycoproteins so its failure to neutralize was not unexpected. Therefore, the chimera should be very useful for screening samples for neutralizing antibodies and discriminating between those that neutralize as compared to those that just bind.

Table 5

Neutralization of chimeric virus growth in Huh 7 cells[1]

	Number of foci[2]		
Virus dilution	Fetal Calf Serum (Boyt)	Anti-HCV HVR1	Anti-BVDV
Undiluted	191	298	0
Dilution (1:10)	23	43	0

1. Huh 7 cells were used for infection but the virus had been grown in EBTr (A) cells.

2. Foci stained with chimp 1530 anti-HCV and visualized by immunofluoresence microscopy.

Table 6

Neutralization of chimeric virus growth in Huh 7 cells[1]

Number of foci[2]				
Fetal Calf Serum (Boyt)	Anti-HCV CH 1530	Anti-HCV H79	Anti-HCV CH 3001	Anti-HCV CH 1494
46	37	0	12	38

1. Huh 7 cells were used for infection but the virus had been grown in EBTr (A) cells; viruses were undiluted.
2. Foci stained with chimp 1530 anti-HCV and visualized by immunofluoresence microscopy.

DISCUSSION

A chimeric genome consisting of HCV structural genes and BVDV nonstrucural genes and untranslated regions was able to replicate in cell lines of bovine and human origin. The HCV glycoproteins and core protein were efficiently expressed from this genome. Virion particles incorporating the chimeric genome were formed only in the presence of an endogenous BVDV helper virus that provided E1 and/or E2 BVDV glycoproteins to each infectious particle. In the presence of helper virus, this chimera replicated to high titers and significant amounts of HCV glycoprotein were released from the cells. Whereas the BVDV glycoproteins are believed to mediate entry into the bovine cells, the HCV glycoproteins on the virions are believed to mediate entry of the chimeric virus into cultured hepatocytes (Huh 7 cells) where the genome replicated via the BVDV non-structural proteins.

Since the chimeric virus replicated to such high levels and such large quantities of HCV

glycoproteins were synthesized, it would be feasible to test purified chimeric virions as a candidate inactivated vaccine. Purified chimeric virions can be tested first in mice and if antibody to HCV is produced, the virions will be tested in chimpanzees to determine if the candidate vaccine is efficacious. The fact that virions grown in EBTr(A) cells were able to infect Huh 7 cells and were neutralized by some anti-HCV positive plasmas (Table 6) suggests that such chimeric viruses could be used to screen for neutralizing antibodies to HCV as well as to screen other cell lines for HCV receptors. The infectivity of the chimera proves the principle that HCV-BVDV chimeras can serve as a useful tool for studying the molecular biology of HCV. The glycoprotein genes from the five other genotypes of HCV can be similarly inserted into the BVDV backbone in order to provide an assay for antibodies to each genotype. Additional chimeras are being constructed in which the core protein of BVDV is included so that only the glycoproteins of HCV are introduced. If BVDV core is critical for encapsidation of the RNA, it may be possible to generate chimeric viruses in the absence of helper. It will also be revealing to determine if the HCV contribution to the chimera can be localized to either E1 or E2 alone. Such a chimera will be tested for its ability to infect EBTr(A) and Huh 7 cells. These studies will help determine the relative importance of E1 and E2 for infection of Huh 7 cells and may define any association with the BVDV glycoproteins. In addition, chimeras in which the BVDV nonstructural genes such as p7 or NS4B or NS5A are replaced with the corresponding genes of HCV may also be generated to

determine if they are functional in cell culture.

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